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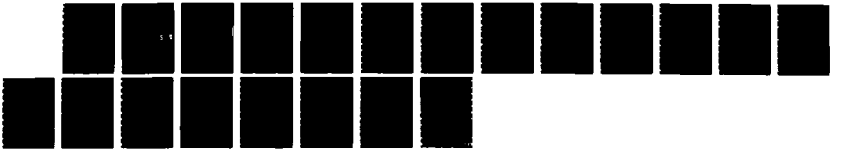

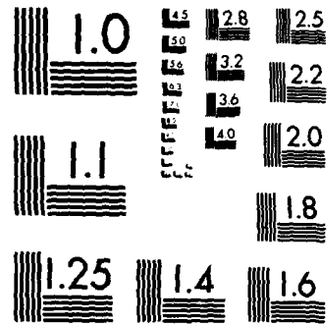

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EXPERIMENTAL TREATMENT FOR BURN VICTIMS IN FIELD HOSPITALS

Annual Summary Report

I.V Yannas and E.M. Skrabut

September, 1984

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Various modifications of the freeze drying process for Stage 1 artificial skin have been attempted and it appears that one of these modifications may prevent pore collapse. The parameters of the skin lymphocyte reaction were studied and it was concluded that additional effort is required before the assay can be shown to be reproducible. Epidermal (basal) cells can be cryoprotected to varying degrees at -80°C by dimethyl sulfoxide and glycerol.

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FORWARD

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In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publicaton No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

1. Method for manufacturing artificial skin in dry state.

Currently Stage 1 artificial skin is a two-layer membrane which is stored in an isopropanol-water solution. Storage in the dry state would be preferable since a) collagen fibers undergo hydrolysis at a slow but non-zero rate over several months of storage in that medium and b) the package containing a dry membrane weighs only a small fraction of the weight of the package containing the membrane in the alcohol solution.

Two procedures are currently pursued in parallel to produce a dry membrane: a) freeze drying of the wet membrane and b) development of a process for crosslinking the membrane with gaseous glutaraldehyde, thereby avoiding immersion of the membrane into an aqueous solution altogether.

a. Freeze drying of the wet membrane.

Introduction. The artificial skin ("Stage 1") is currently produced by first forming a slurry of collagen and chondroitin 6-sulfate which is subsequently cast in pans and freeze dried to give a highly porous solid. The latter is first treated in a vacuum oven, then covered with a thin layer of silicone rubber, and is then immersed in an aqueous acetic acid solution of glutaraldehyde. Finally, the glutaraldehyde is rinsed off with water and the membrane is stored in 70% aqueous isopropanol in a sealed polyethylene bag and refrigerated until ready to use.

Efforts to dehydrate the two-layer membrane by freeze drying have produced two undesirable effects. First, the membrane curls up due to the fact that the collagen-glycosaminoglycan (CG) layer contracts somewhat during drying while the silicone layer does not. Second, the CG layer loses its porosity, apparently by pore collapse during freeze drying, and the membrane becomes unsuitable as a graft for full-thickness excised wounds in guinea pigs. The work described below was a preliminary attempt to circumvent these two problems. Freeze drying was attempted a) at different shelf temperatures and b) from different liquid media, in order to detect empirical trends that may assist in formulating useful quantitative models of pore collapse. Such a model could eventually be used to redesign the process.

Dr. B. Pruitt has suggested to us that the solution of the problem of pore collapse may lie in the use of appropriate cryoprotective agents. This suggestion, together with others that our data will lead us to, will be tested in future work.

The CG membranes were prepared as described previously [1] except that no silicone layer was bonded onto their surface. This simplification in the fabrication procedure allowed separating out effects due to curling up of the membrane during freeze drying from pore collapse. It is possible to bond the silicone layer later in the fabrication process without apparently affecting performance of the membrane as a graft.

Experimental. Membranes that had been fully processed were freeze dried at two levels of the shelf temperature of the freeze drier: -40 C and -100 C. Three liquid media were used during freeze drying: distilled water, 10% aqueous isopropanol and 70% aqueous isopropanol. The pore structure of experimental membranes was viewed by scanning electron microscopy at 100X and at 300X using procedures that have been published [2].

Samples for measurement of the peel strength (the force per unit length required to separate the silicone layer from the CG layer) were prepared by first freeze drying the CG membrane according to one of the experimental procedures, then bonding the dry CG sheet onto a section of silicone rubber film (Dow Corning, Midland, MI) which had been previously coated with a layer of a moisture-curing silicone medical adhesive (Silicone Medical Adhesive A, Dow Corning, Midland, MI). The silicone layer was separated from the CG layer using a scalpel and the two separated edges were attached to the grips of an Instron Tensile Tester and pulled apart.

Wound contraction was studied by grafting guinea pigs with each of the materials manufactured. Grafts (3.0 x 1.5 cm) stored in 70% isopropanol were cut under sterile conditions and placed in normal saline in a Whirl Pak bag (American Scientific Products, Bedford, MA). The grafts were shaken and massaged to remove the 70% isopropanol from the pores, and then were placed in another Whirl Pak bag of sterile normal saline and the massaging and shaking was repeated. The grafts were placed in fresh normal saline and were ready for grafting.

White, female, Hartley guinea pigs Charles River Laboratories, Inc., Wilmington, MA) weighing 300-400g each were shaved and dehaired with Nair (Carter Products, Division of Carter-Wallace, Amer. Hosp. Supply, Irvine, CA). Each guinea pig was given an intramuscular injection of Keflin in the hind quarter (intramuscular tetracycline, Pfizer, 0.035 mg in 1 ml, 0.1 mg/kg) as a prophylactic antibiotic, approximately one half hour before surgery. The anesthesia was 1-3% Fluothane (Metofane, Pitman-Moore, Washington Crossing, NJ) in oxygen. A full thickness excision (1.5 x 3.0 cm) was made up to but not including the panniculus carnosus on the left side of the back of the guinea pig. A graft was placed on the wound with the collagen-GAG surface against the wound bed, and was sutured in place with ten black monofilament nylon sutures.

The animals were bandaged after surgery with sterile gauze and a double layer of Elastoplast bandages (Beiersdorf, Inc., South Norwalk, Conn.)

The contraction kinetics of the grafted wounds was followed at three or four day intervals [3,4]. The animals were unbandaged, observed and photographed. A Nikkormat camera with Macro Nikkor lens, 55 mm, f3.5 (Nikon, Japan) was used to photograph the grafts with Kodachrome ASA64 color slide film (Eastman Kodak, Rochester, NY). The slides were projected and the pink area of the graft was traced onto white paper. The pink area is the area underneath the silicone layer which has not epithelialized or contracted. Thus, the pink area is a measure of both the contraction and epithelialization. Areas were calculated by cutting out and weighing the tracings.

The 50% closure day or wound contraction half-life is the day post surgery when the pink area has been reduced to one-half of its value at the time of grafting.

Results. The table below lists the preliminary results obtained to date:

TABLE 1. Effect of terminal freeze drying conditions on porosity

Shelf temp., C	Liq. medium	Porosity	Macroscopic properties of dry foam
-40	water	Poor (closed)	No fractures
-100	water	fair to good	Macroscopic fractures
-100	70% isopropanol	good	No fractures
-100	10% isopropanol	good	No fractures

There were no remarkable differences in porosity between membranes which were produced by freeze drying the CG slurry (the first part of the current procedure for fabricating CG membranes) and membranes which were produced by the last two experimental freeze drying procedures listed in Table 1. Examination of the former membrane (control) and of the latter two membranes with the scanning electron microscope at 100X and 300X showed no apparent differences in mean pore size (approx. 100 μm).

The bond formed between the silicone layer and the CG layer was equally strong in specimens produced by the procedure listed as the last entry in Table 1 as in control specimens (which are fabricated by the current procedure). The peel strength of control specimens was found to be 7.9 ± 1.9 g/cm compared to the value 10.2 ± 1.3 g/cm for the experimental membranes.

These results suggested that the experimental bilayer material was suitable for grafting on animals. Experimental animals were grafted and were unbandaged at 3-day intervals, and the remaining wound area was measured. Control animals showed a wound contraction half-life of 19 ± 2 days while the experimental animals (grafted with membranes produced according to the last entry in Table 1) showed a half-life of 18 days. This result is based on observations on three animals only.

Animals were sacrificed on days 7,14,21 and 65 and histological sections were prepared and studied. Study of wound tissue which had been grafted with experimentally freeze dried material indicated a higher incidence of undigested CG fibers by day 14 than has been observed with control grafts.

There was less cellular infiltration into the experimentally processed grafts than into the controls at day 14. Also, there appeared to be more inflammation than in the 14 day control. However, the inflammatory reaction observed was still mild. The experimental material appeared to be much thinner than the control at day 14. By day 21, the experimentally processed grafts were well infiltrated with cells (similar to the degree of infiltration observed at day 14 of the control). At this time, there appeared to be many multi-nucleated giant cells associated with the CG fibers.

Discussion and conclusions. The experimental freeze drying procedures described in Table 1 represent a partial solution to the problem of dehydration of a wet graft without significant loss of its porosity. Although the experimental work is preliminary it suggests that isopropanol may be a useful additive in the terminal freeze drying process. The physicochemical properties of the ideal additive remain to be defined pending gathering of additional empirical data. However, it remains questionable whether the optimal substance should be a very efficient melting point depressant or not.

Although the porosity as viewed by scanning electron microscopy is apparently preserved following some of the experimental freeze drying procedures, it is possible that the "degree of subdivision" of the fibrous components of the CG membrane may not be preserved so well. If, for example, terminal freeze drying has the effect of causing collapse of discrete fibrils into a massive fibrous bundle (while leaving the average pore size relatively constant), the resulting massive fibers

would be digested in vivo at very low rates. Such a hypothetical mechanism could explain the histological finding that CG fibers remain undigested over longer periods of time when experimentally processed grafts are compared with controls. It is conceivable that systematic use of the SEM at greater magnification could provide some semiquantitative information on the degree of subdivision of the fiber before and after terminal freeze drying.

b. Use of gaseous glutaraldehyde as a crosslinking agent.

Introduction. Previous early results obtained with dry CG membranes that had been exposed to glutaraldehyde vapor suggested that such exposure leads to crosslinking of the CG matrix. However, this early work suggested the need for a screening study which would indicate the critical variables that affect the extent of the reaction.

Results. The two variables studied were temperature and light. The results appear on Table 2 and 3 below. Each of the M_c results is the average of 5 determinations.

TABLE 2. Effect of temperature on the extent of reaction between CG and gaseous glutaraldehyde

Temp., C	Light/Dark	Average mol. wt. between crosslinks, M_c
-1	light	17,600 ± 2,300
23	light	14,400 ± 2,900
35	light	6,100 ± 1,700

TABLE 3. Effect of light on the extent of reaction between CG and gaseous glutaraldehyde

Temp., C	Light/Dark	Average mol. wt. between crosslinks, M_c
26	Dark	4,400 ± 900
26	Light*	9,000 ± 1,800

* An ultraviolet lamp in the setting of a sterile verical laminar air-flow bench was used.

Table 2 shows that the extent of the reaction increases significantly with temperature as shown by a significant decrease in the average molecular weight between crosslinks, M_c. The quantity Mc is inversely proportional to the crosslink density.

Table 3 shows that light has a significantly negative effect on the extent of reaction.

Discussion. The results obtained suggest that control of temperature and of ambient UV radiation are mandatory in any experimental study of this reaction.

We are currently constructing a vapor-phase reactor in which the temperature, the ambient light and the intensity of convection in the vapor phase can be controlled. In addition, the humidity will be continuously monitored by use of a hygrometer.

2. Method for hydrating the CG membrane within several minutes using standard hospital solutions.

Work on this topic has been suspended temporarily pending a successful demonstration of a process modification for fabrication of Stage 1 artificial skin in the dry state (see Section 1 above).

3. Develop biophysical methods for reducing the immunogenicity of heterologous epidermal cell preparations.

a. Introduction. Work in this area has the objective of developing a donor-independent Stage 2 artificial skin. The latter is a Stage 1 membrane (silicone bonded to CG) which has been seeded with basal epidermal cells [5]. The cells used as seed have so far been autologous cells [5]. The result is partial regeneration both of the dermis and the epidermis on the site of the full-thickness excised skin wound on guinea pigs [5]. The regeneration is partial in the sense that the resulting integument has no hair or adenexa. our effort has therefore focused on the preparation of a population of basal epidermal cells wich would be so weakly immunogenic as to be useful for inoculating Stage 1 membranes and obtaining the full "Stage 2" response without evidence of rejection over at least several months.

The preliminary strategy consisted in first preparing a standard basal cell preparation from guinea pig skin, treating such preparations in various biophysical modes, developing an assay (the skin lymphocyte reaction, SLR) to determine the effects of such treatments on the immunogenicity of the basal cell population and eventually grafting full thickness skin wounds on guinea pigs with basal cell populations that showed a sufficiently weak SLR reaction.

Most of our effort was directed towards development of a highly reproducible SLR assay. This goal turned out to be somewhat elusive.

b. Experimental. (i) Standard basal cell preparation. The procedure used has been described elsewhere [5].

(ii) Use of Percoll gradient. The cell suspenion was layered onto a Percoll (Pharmacia, Piscataway, NJ) gradient (a 1:1 ratio of isotonic Percoll: DMEM, Dulbecco's Modified Eagle's Medium was used) and was centrifuged at 1000xg for 30 min. The result was a band of cells which had migrated slightly into the gradient and a cell pellet. The cells in the band and pellet were isolated and washed once with DMEM. Unfractionated cells were used as a control in this study.

(iii) Development of skin lymphocyte reaction (SLR) assay. Peripheral blood lymphocyte isolation. Peripheral blood lymphocytes are isolated from the guinea pig according to a modification of a previously published method [6]. By this method, whole blood is witdrawn by cardiac puncture into a heparinized syringe. The guinea pig is anesthetized during this procedure. Once the blood is in the laboratory, it is diluted 1:4 with phosphate buffered saline, PBS (without calcium and magnesium) containing 5 mM EDTA. The diluted suspension is centrifuged for 1 minute at 1000 x g to separate the RBC and WBC components from the platelets. The cell pellet is resuspended in phosphate buffered saline containing 5 mM EDTA and 0.6% dextran. The dextran promotes the agglutination of erythrocytes, causing them to settle out of the cell suspension more quickly. The suspension is allowed to settle for 30 to 45 min at room temperature and the WBC- rich supernatant is then removed.

The lymphocytes are purified from WBC suspension by centrifuging over a density step gradient (density = 1.107 g/ml). The original gradient which was used for its separation was a Percoll gradient. However, not even the erythrocytes settled through the gradient after centrifugation. Attempts were made to improve the results by decreasing the density of the Percoll. The best results were obtained with a density of 1.065 g/ml. This gave a difuse band of cells which became more distant after allowing to stand overnight. Increased centrifugation time did not give a more defined band. These lymphocyte preparations were contaminated with large amounts of RBC's and PMN's. It is hypothesized that the Percoll decreased the density of the cells by absorbing to their surface.

A ficoll-metrizoate gradient (density = 1.107 g/ml) was substituted for the Percoll. This gradient was underlayed below the cell suspension. Two ml of gradient were used for each 5 ml of cell suspension. The cells were then centrifuged into the gradient at 400 x g for 2.5 to 3 hrs. This resulted in a well defined band of lymphocytes. The band was removed with a Pasteur pipette and washed with PBS. The cell are then resuspended in

DMEM (+10% Fetal Calf Serum, FCS) and adjusted to a cell concentration of 1 x 10^6 ml. The cell suspension is checked for purity by staining with Wright-Giemsa.

Peritoneal exudate cell isolation. An alternative method of isolating lymphocytes was from peritoneal exudate by the method of Rosenstreich, et al.[7]. This was accomplished by injecting 20 ml of sterile mineral oil in the peritoneal cavity of a guinea pig. Three days later, the cells are harvested in one of two ways. With the first method, sterile PBS is injected into the peritoneal cavity and then withdrawn. This is repeated one or two times. This method has the advantage that no surgery or anesthesia is required. However, the yield of cells is low. The second method requires that the animal is placed under anesthesia. A small incision is made in the abdomen; PBS is injected into this and then withdrawn. Although more exudate is withdrawn by this method, it has the disadvantage that the preparation is contaminated with RBC's. The exudate cells are then washed with DMEM (+1% Guinea Pigs Serum, GPS) and suspended in two ml of GPS. A column is prepared by packing a 20 ml syringe with nylon wool, sterilizing it, and flushing with 40 ml of warm DMEM. The cell suspension is then applied to the column and allowed to percolate into it. The column is then incubated at 37°C for 20 min. The column is then flushed with DMEM (+10% GPS). The eluted cells are washed with serum free media and resuspended in DMEM (+10% FCS).

Lymph node lymphocyte isolation. Lymph node lymphocytes have been isolated from lymph nodes located at various areas of the guinea pig. These are obtained most easily from the cervical lymph node. The guinea pig is placed under anesthesia and an incision is made in the neck. The cervical lymph node is then removed and kept in cold PBS. The fat is trimmed from the node and then the node is minced with a scalpel and forceps. The cells are then suspended in DMEM (+10% FCS) and filtered through cheese cloth to remove large sections of tissue. The cell concentration is then adjusted by adding DMEM. This method has the largest yield of lymphocytes.

Measurement of immunogenic response. The assay consists in measuring the immunogenic response by making use of the fact that stimulated lymphocytes undergo blast transformation and thereby experience increased DNA synthesis. The extent of the reaction is determined by measuring the uptake of a labeled DNA precursor, ^{3}H-thymidine, over a fixed period of time in a control population and in a stimulated population. We have used the "response index", the ratio of stimulated DNA synthesis to unstimulated DNA synthesis, as a measure of the vigor of the reaction. A response index of 1 indicates no signification stimulation.

Preliminary tests of activity of lymphocyte preparations were done by culturing the lymphocytes with either phytohaemagglutinin (PHA), which stimulates T lymphocytes, or pokeweed mitogen (PWM), which stimulates B lymphocytes.

C. Results. Lymphocyte responsiveness was found to vary not only from one animal strain (Hartley) to another strain (English) and from animal to animal within the same strain but also from day to day in the same animal. Such variability made it very difficult to obtain satisfactory reproducibility in value of the response index.

Peripheral blood lymphocyte (PBL) isolations involve a day-long prcedure and do not yield enough cells for an assay with many duplicates. A reproducible response index for PHA-treated Hartley cells is about 45 (compared to a response index of 1 for untreated cells), and that for English cells is about 120, indicative of a very responsive population. However, in a mixed lymphocyte reaction (MLR) involving culturing of cells from two different animals, no response was detected (response index, 1). When subjected to cryopreservation, 14 days at -70°C, the PHA-treated lymphocyte activity was reduced 15-fold. Further work was not done with PBL because of their limited availability and their lack of response in the MLR.

Peritoneal exudate lymphocyte (PEL) preparations do not yield a large number of lymphocytes. The response index of PHA-treated cells was 9.9 in one experiment, and 1.7 for PWM-treated cells. In an SLR involving Hartley lymphocytes and a standard preparation of heterologous basal cells (Hartley), a response index of 1.5 was measured. This result has not been reproducible.

Lymph node lymphocytes (LNL) are readily accessible and yield enormous quantities of pure lymphocytes. The highest response index for PHA-treated Hartley cells has been 3.4, and 13.3 for English cells; response to PWM has been 1.8 for both cell types. The highest response recorded in an SLR involving a standard basal cell preparation has been 1.7. In an SLR assay of mature Hartley epidermal cells with English lymphocytes a response index of 5.6 was recorded; with Hartley lymphocytes the index was 4.9. These results have not been reproducible.

Percoll separations of epidermal (basal) cells yielded a band and a cell pellet. The cell pellet had a response index of 2.4 in the SLR while the unfractionated basal cell preparation and the band in the Percoll gradient had a response index of 1 (no significant response).

d. Discussion. The major problem with the SLR is lack of reproducibility. This occurs with all three sources of lymphocytes and with both strains of guinea pig. Studies will be conducted to determine the variability of fixed pairs of guinea pigs. When a set of "responding" guinea pigs is found, this will be subjected to the SLR repeatedly to determine if the activity remains at a high level.

Another possibility which may add to the non-reproducibility of the SLR is contamination with Langerhans cells (LC). These cells are isolated along with the basal cell preparation and may themselves be stimulated by the lymphocyte in the assay system. At this time there is no mechanism of determining the amount of activity observed which was due to the LC population. Future studies will be conducted to quantify the LC content of the skin preparation and determine if there is an effect on the SLR.

The SLR assay may be species dependent. In order to test for this possibility, the SLR will be setup with human cells. This will determine if the problem is in the assay itself or is a result of the species being utilized. Human cadaver skin will be obtained and used only if it is less than 24 hrs old and has not been treated with a cryopreservative. The human lymphocytes will be obtained from the peripheral blood of normal volunteers by isolation with density gradient centrifugation techniques. Both SLR and MLR will be utilized to determine the reactivity of the human cell preparations.

4. Develop an efficient procedure for cryopreservation of the basal cells with dimethyl sulfoxide and glycerol.

Introduction. This part of our effort emphasized cryopreservation at -80° C, i.e. at mechanical refrigeration temperatures rather than at liquid nitrogen (-196° C) temperature where significant inconveniences and high cost are serious disadvantages.

Experimental. Epidermal (basal) cell populations were harvested as previously reported [5]. The cell suspension was assayed for cell number and viability using a Trypan Blue dye exclusion technique. The suspension was then adjusted to a concentration of 1×10^6 viable cells/ml. One ml aliquots of the suspension were transferred to 15 ml centrifuge tubes prior to addition of the cryopreservative.

Dimethyl sulfoxide (DMSO) was added to aliquots of the cell suspension until the DMSO concentration was as high as 2.1 M. Glycerol was added to similar aliquots until its concentration in the cell suspension was as high as 7.8 M.

The cryopreservatives were added to cell suspensions which were maintained either at 4° C or at 22° C. Following addition of the cryopreservative at 4° C, the suspension was allowed to remain at 4° C for one half hour before being placed in a freezer maintained at -80 C for 3 to 7 days. When the cryopreservative was added at 22° C, the cell suspension was then incubated at 37° C for one-half hour. Following this incubation, the cells were then frozen to -80° C for 3 to 7 days.

After a period of 3 to 7 days at -80° C the cell suspensions were thawed by immersion in a circulation of water bath maintained at 37° C.

The cryopreservative was removed as follows. Eight ml of sterile 1.6% NaCl solution was added to each vial. The suspensions were then replaced in the environment they occupied before freezing: either a 4°C water bath or 37° C incubator, and remained there for one half hour. Centrifuging at 500 xg and 4°C for ten minutes caused settling out of the cells so that eight ml supernatant could be replaced by eight ml of 0.9% NaCl. The cells were resuspended and were again placed in their respective environments for one half hour before an additional cycle of centrifugation. All supernatant was then removed and the pellet was resuspended in five ml Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum. One half ml samples were removed from each vial for Trypan Blue dye exclusion determination of viability.

Results. Cell viability results following freezing, thawing and removal of cryopreservative are presented in Table 4.

TABLE 4. Epidermal (basal) cell viability following freeze-thawing cycling (-80°C for 3 to 7 days)

Cryopreservative	Molarity	Temperature of incubatiion	%Cell Viability
Glycerol	0	4	12 ± 5
"	0.5	"	16 ± 2
"	1.0		15 ± 2
"	1.5	"	12 ± 2
"	2.0	"	7 ± 3
"	2.5	"	11 ± 4
Glycerol	0	37	7 ± 2
"	1.5	"	52 ± 7
"	3.1	"	38 ± 4
"	4.6	"	16 ± 4
"	6.2	"	1 ± 1
"	7.8	"	0
DMSO	0	4	6 ± 2
"	1.1	"	71 ± 9
"	1.4	"	76 ± 10
DMSO	0	37	0
"	0.7	"	9 ± 2
"	1.1	"	27 ± 6
"	1.4	"	40 ± 8
"	1.8	"	41 ± 5
	2.1	"	25 ± 3

The results show that the temperature at which the cryopreservative is added to the epidermal cell suspension strongly affects the freeze-thaw viability in glycerol-treated more than in DMSO-treated cells. In particular, cell viability as high as 52% was obtained when 1.5 M glycerol was added at 37°C compared to viability of 12% when glycerol at the same molarity was added at 4°C. In the case of DMSO, Cell viabilityies of 76% could be obtained when 1.4 M DMSO was present at 4°C; this viability was reduced to 40% when the same concentration of DMSO was present at 37°C.

Discussion. The difference in degree of cryoprotection offered when glycerol is added at the two different temperature levels suggests that the cryopreservative may have been prevented from entering the cells maintained at 4°C. Such a result could conceivably be explained by hypothesizing that glycerol is transported across the cell membrane by a biologically active process, such as facilitated diffusion or active diffusion, and that such a process slows down significantly when the temperature drops.

The difference in the degree of cryoprotection offered by DMSO at the two temperatures of addition is not as large. In this case, however, there is greater protection at the lower temperature. This would indicate that the transport of DMSO across the membrane is not an active process. It can be hypothesized that at elevated addition and incubation temperatures, some damage occurs to the cell membrane due to the exothermic reaction of dissolving DMSO.

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